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Genetic Analysis of vancomycin-resistant gram-positive cocci isolated from wild songbirds

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GENETIC ANALYSIS OF VANCOMYCIN-RESISTANT GRAM-POSITIVE COCCI ISOLATED FROM WILD SONGBIRDS

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ABSTRACT

The antibiotic vancomycin was developed by Eli Lilly in the 1950s in response to the growing number of *Staphylococcus aureus* infections that were resistant to penicillin. Vancomycin was not widely used at the time because of its high toxicity. However, use of vancomycin in the United States has increased dramatically since the 1980s because of the emergence of methicillin resistant *Staphylococcus aureus* (MRSA) and *Enterococcus* species. There are three known mechanisms for vancomycin resistance: 1) target site modification by *van* genes, 2) biofilm formation, and 3) bacterial cell wall thickening. Of these mechanisms, target site modification is the most common. In this study, we analyzed cultivable highly vancomycin-resistant Gram-positive bacteria from the saliva of migratory songbirds, which were captured at the bird banding station at Fort Hays State University in previous work. Individual bacterial isolates were identified by partial 16S rRNA sequencing. The majority of the identified bacteria were *Staphylococcus succinus,* with the majority being isolated from American Robin, which is commonly found in all of North America. Some of these bacteria carry *van*A, *van*B, and *van*C genes and also have the ability to form biofilms. One of the *van* gene-carrying isolates is also coagulase-positive which is also considered a strong virulence factor. Given the wide range of the American Robin and ease of gene transfer between Gram-positive cocci, we postulate that these organisms could serve as a source of vancomycin resistance genes in human pathogens.

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PREFACE

The literature cited, tables, and figures of this thesis were written in the style of the American Society for Microbiology, where it will be submitted for possible publication in the Journal of Applied and Environmental Microbiology.

INTRODUCTION

Vancomycin

Vancomycin, which is classified as a member of the glycopeptide antibiotic group, was isolated from *Streptomyces orientalis* by Eli Lilly and Company in 1952. They started clinical trials for vancomycin in human subjects and concluded that it was very effective against penicillin-resistant bacteria. Unfortunately, they also detected high toxicity of vancomycin to humans. The observed symptoms of vancomycin toxicity included ototoxicity, venous irritation, chills, and rash, which were thought to be largely due to impurities of vancomycin in those early preparations. Rapid infusion of vancomycin also caused "red man" or "red neck" syndrome which is characterized by a combination of erythema, pruritis, hypotension, and angioedema. By contrast, methicillin, the first semisynthetic penicillin, was less toxic than vancomycin, so vancomycin was reserved only for patients with severe infections (Levine, 2006).

The target site of vancomycin against bacteria is the dipeptide, D-Ala-D-Ala, on the pentapeptide within the peptidoglycan layer. Transpeptidase produced by bacteria recognizes the dipeptide (D-Ala-D-Ala) at the end of the pentapeptide chain, cleaves off the terminal alanine, and joins the remainder to the branch of a stem peptide from an adjacent polysaccharide chain. In another words, the transpeptidase is responsible for finalizing bacterial cell wall synthesis. Inhibition of this process by vancomycin leads to the destruction of the bacterial cell by osmotic pressure (Figure 1).

Vancomycin Resistance - *van* Genes

One of the major resistance mechanisms bacteria use against vancomycin is the vancomycin-resistance genes, which are called *van* genes. There are six well characterized *van* genes: *van*A, *van*B, *van*C, *van*D, *van*E, and *van*G. Bacteria carrying *van*A, *van*B, and *van*D confer high levels of Minimum Inhibitory Concentration (MIC) against vancomycin. MIC is the term used to describe the lowest amount of antibiotic required to kill or inhibit organisms. Furthermore, *van*A and *van*B achieve very high vancomycin resistance (MIC= up to 1000 mg/L) (Courvalin, 2006). It has also been shown that the *van*A gene also has the ability to confer high resistance to another member of the glycopeptide antibiotic group, teicoplanin (MIC= up to 512 mg/L) (Courvalin, 2006). What vancomycin resistant organisms do is to change the sequence of the D-Ala-D-Ala to D-Ala-D-Lac or D-Ala-D-Ser to prevent vancomycin binding (Figure 2). This decreases the affinity between the vancomycin and the dipeptide. The affinity between vancomycin and the precursor dipeptide D-Ala-D-Lac (for *van*A, *van*B, and *van*D) is 1000-fold lower than for the precursor dipeptide D-Ala-D-Ala. In the case of *van*C, *van*E, and *van*G, the affinity between vancomycin and precursor dipeptide D-Ala-D-Ser is 6-fold lower than for the precursor dipeptide D-Ala-D-Ala (Hong et al, 2008). Due to the much lower affinity, *van*A, *van*B, and *van*D genes produce high levels of vancomycin resistance. Furthermore, the expression of *van*A and *van*B genes is inducible, and is the most frequently encountered type of glycopeptide resistance (Courvalin, 2006). The genes *van*A, *van*B, *van*C, *van*D, *van*E, and *van*G, are responsible for ligase function, which connects the amino acids and synthesizes dipeptide in the pentapeptide of

peptidoglycan layer. These vancomycin-resistance genes are only part of a key set of genes required to change the sequence of the dipeptide within the pentapetide. In other words, the existence of those *van* genes, *van*A, *van*B, *van*C, *van*D, *van*E, and *van*G, alone cannot change the sequence of dipeptide. The vancomycin-resistant bacterial strains usually carry a cluster of resistance genes which function to change the sequence of dipeptide targets. The main resistant genes which achieve the change of dipeptide sequence are vanH, which produces a dehydrogenase that converts pyruvate to D-Lac, *van*A (or other ligase producing gene such as *van*B) which catalyzes the formation of an ester bond between D-Ala and D-Lac, and *van*X, a D,D-dipeptidase which hydrolyzes the dipeptide D-Ala-D-Ala synthesized by the host Ddl ligase enzyme (Figure 3) (Courvalin, 2006).

These are common genes in vancomycin-resistance operons, but the resistance genes themselves in each operon are different. For instance, the *van*B gene operon has *van*W gene of unknown function, which the *van*A operon does not carry (Figure 4). However, the most significant difference between the bacteria carrying the group of *van*A and *van*B operon and the *van*D operon is the control of expression. Both the *van*A and *van*B operons are inducible, while the *van*D operon is constitutive (Depardieu et al, 2004a).

Another difference between the group of *van*A- and *van*B- versus *van*D- type strains is lack of D,D-dipeptidase activities. The v*an*A- and *van*B-type strains require *van*X activity to hydrolyze the dipeptide D-Ala-D-Ala synthesized by the host Ddl ligase, but the *van*D-type strains do not require the *van*X activity since the chromosomal *ddl* gene is

mutated, which means absence of an active D-Ala:D-Ala ligase. Consequently, the *van*D-type strains should grow only in the presence of vancomycin since they rely on the inducible resistance pathway for peptidoglycan synthesis (Figure 5) (Depardieu et al, 2004a).

Vancomycin Resistance-Biofilms

Another major vancomycin-resistance mechanism in some bacteria is biofilm formation, which slows the penetration of antibiotics to the cytoplasm of the bacterial cell. (Stewart, 2002 and Dunne et al, 1993). Biofilm formation is also known as one of the most threatening virulence factors researched by microbiologists because biofilm-embedded bacteria are more resistant to antimicrobial agents and often cause chronic infections and sepsis in immunocompromised patients (Schlag et al, 2007). A major complication of biofilm formation is device-related infections, which are serious clinical problems today. The majority of hospital patients undergo procedures for the insertion of foreign devices, such as catheters, artificial heart valves, and joint replacements. Device-related infections cause significant problems since the biofilm-embedded bacteria have resistance against many antimicrobial agents (Stewart, 2002).

In the case of *Staphylococcus aureus,* the biofilm is created by polysaccharide intercellular adhesin (PIA) which leads the bacteria to attach to, and accumulate on, host tissues. Some bacteria involve other components to form biofilm. The control of biofilm formation is basically regulated by the quorum sensing mechanism, which allows bacteria to detect the density of their own species. Quorum sensing has also been shown to control

the stability of the three-dimensional biofilm structure (Bjarnsholt and Givskov, 2007). There are several advantages to the formation of biofilm by bacteria. First, bacteria embedded within biofilm are basically surrounded by exopolymer substance matrix (EPS) which creates pools of genes allowing for genetic acquisition and exchange by horizontal transfer, which leads to alteration of gene expression of bacteria within the biofilm, allowing them to become resistant to antibiotics. The EPS protects the bacteria inside the biofilm from attack by antibiotics. Furthermore, a decrease of bacterial growth within the biofilm increases resistance to antibiotics, enhancing survivability. (Clutterbuck et al, 2007). Scientists have researched the mechanisms of biofilm formation because many genes are involved in the process. The genes related to biofilm formation are different between various bacterial strains, so that biofilm formation is a very complex process.

Quorum sensing is also related to autolysis, which is responsible for the destruction of the cell wall and subsequent cell lysis. Biofilm formation requires extracellular DNA (eDNA) for stability and development. Autolysis destroys the bacterial cell wall, leading to the release of eDNA from the dead cell, which is subsequently used for construction of the biofilm by the living cells that remain (Rice et al, 2007). Autolysis also plays a role in cell wall thickening, which is another mechanism used by some bacteria to resist vancomycin (Gazzola and Cocconcelli, 2008).

The cell wall thickening process basically thickens the peptidoglycan layer in the bacterial cell. For Gram-positive bacteria, the peptidoglycan layer is composed of glycan chains that are constructed by alternating N-acetylmuramic acid (NAM) and N-

acetylglucosamine (NAG) subunits. Tetrapeptides, which extend from the NAM subunits of the glycan chains, act as anchor points for peptide interbridges, allowing the glycan chains to be linked together to form a three-dimensional meshwork (Hiramatsu, 2001). According to Sieradzki and Tomasz, some vancomycin-resistant *Staphylococcus aureus* (VRSA) strains stop the autolysis process in the presence of vancomycin, so the cell wall becomes thickened (1997). In this work, investigators cultured VRSA in growth media containing vancomycin to measure the decrease of the vancomycin concentration during bacterial growth. According to these experiments, the concentration of the vancomycin in the media decreased gradually as the bacterial growth increased (Sieradzki and Tomasz, 1997). According to Cui et al (2006), the thickness of cell wall for vancomycin-intermediate *Staphylococcus aureus* (VISA) strain Mu50, was 35.02 nm, which is 1.64 times thicker than the cell wall of vancomycin-susceptible *Staphylococcus aureus* (VSSA). It was demonstrated that vancomycin clogs the bacterial peptidoglycan layer, which prevents further penetration of subsequent vancomycin molecules (Cui et al, 2006). The thickening cell wall correlates with an increase of vancomycin MIC. These investigators also detected that the vancomycin MIC of those strains decreased under the vancomycin-free environment (Cui et al, 2003). Some of the VRSA strains lost the vancomycin-resistance phenotype under the antibiotic-free condition so these workers postulated that these bacteria somehow use the vancomycin (Cui et al, 2003).

Vancomycin-Resistant Pathogens- VRE and VRSA

Two of the major pathogenic groups of bacteria that exhibit vancomycin resistance are vancomycin-resistant enterococci (VRE) and vancomycin-resistant *Staphylococcus aureus* (VRSA). A certain type of VRE is called vancomycin-dependent *Enterococcus* (VDE) because it requires vancomycin for cell wall synthesis. Vancomycin-resistant strains usually change the amino acid structure of their cell walls to avoid the interaction between vancomycin and the cell wall components, which essentially leads to vancomycin inactivation (Bambeke et al, 1999). In other words, VDE strains cannot survive without vancomycin because of mutation of the *ddl* gene. As mentioned previously, the chromosomal *ddl* gene is responsible for D-Ala:D-Ala ligase function which synthesizes the dipeptide for bacterial cell walls. Mutation of the chromosomal *ddl* gene means inactivation of synthesis of the dipeptide for bacterial cell wall formation. In another words, the bacteria cannot synthesize bacterial cell walls unless other genes synthesize the bacterial cell wall dipeptide, replacing the function of the defective *ddl* gene. Consequently, VDE strains substitute the dipeptide, D-Ala-D-Lac or D-Ala-D-Ser, synthesized by *van* genes for bacterial cell wall synthesis since vancomycin activates *van* genes (Bambeke et al, 1999). Another phenomenon similar to vancomycin dependence in VDE has been reported by Dantas et al who demonstrated some bacteria have the ability to survive on antibiotics as their sole carbon sources (2008).

Another serious vancomycin-resistant organism is vancomycin-resistant *Staphylococcus aureus* (VRSA), which is related to methicillin-resistant *Staphylococcus aureus* (MRSA). Methicillin was introduced in Europe in 1959 and in the United States in 1961, and the first reported case of MRSA was in the United Kingdom in 1961. The

first reported case of MRSA in the United States was in 1968. MRSA is currently known as one of major pathogens in hospitals all over the world (Rice, 2006). According to a meta-analysis report in 2008, the overall mortality rate caused by MRSA infection was over 50%. In two of those analysis cases, it was over 70% (Dancer, 2008). In the United States, 59.5% of ICU patients were infected with MRSA according to the National Nosocomial Infections Surveillance (NNIS) system report for 2004. Furthermore, that number represented an 11% increase of MRSA infections during the period 1998 to 2002 (Rice, 2006). Vancomycin is currently the drug of choice for MRSA infections, but the use induces emergence of VRSA and vancomycin intermediate *Staphylococcus aureus* (VISA) (Jones, 2008).

The first reported case of VRSA was in 1996 in Japan. In the United States, the first four cases of VISA were reported between 1997 and 1999 (Rice, 2006). The mortality rate of the patients who acquired VISA was 63% (Dancer, 2008). A main difference between VISA and VRSA is the MIC. The MIC of vancomycin against VISA strains is 8 to 16 mg /L, whereas in the case of VRSA it is 64 mg/L. Another main difference is the mechanism of resistance against vancomycin. For VISA strains, the cell wall thickens, which causes it to trap the vancomycin molecule in the outer layer of the cell wall, thereby limiting access to the cytoplasmic membrane where the functional targets of vancomycin are located. In the case of VRSA strains, the organisms carry vancomycin resistance genes such as *van*A, which produces an enzyme to change the dipeptide sequence of bacterial cell wall, and involves horizontal transfer of transposons from VRE (Rice, 2006).

Both MRSA and VRSA originally came from *Staphylococcus aureus* which can inhabit the surface of human skin or nasal passages as normal flora. VRSA strains carry vancomycin resistance genes, and MRSA strains carry many antibiotic resistance genes. Many of today's MRSA strains have tolerance to vancomycin. *Staphylococcus aureus* produces many toxins such as α-toxin, staphylococcal toxic shock syndrome toxin (STSST), enterotoxins, and Panton-Valentine leucocidin (PVL). For instance, PVL strains cause specific human infections such as necrotizing pneumonia, where the mortality rate is up to 75%. Furthermore, some of these toxins are enhanced by use of certain antibiotics such as members of the ß-lactamase antibiotic group (Dancer, 2008). Normally, infections caused by *Staphylococcus aureus* are treated by antibiotics, but it is difficult to treat with antibiotics if the infection is caused by MRSA, since MRSA is resistant to many antibiotics. Furthermore, it has been reported that certain MRSA strains are resistant to the newest antibiotic, linezolid (Arias et al, 2008). According to that report, a 52-year-old female acquired a MRSA infection during a surgical procedure, and the hospital prescribed six different antibiotics which are relatively powerful antibiotics. However, the patient died of the infection at day 27 because of multi-organ failure (Arias et al, 2008). There are two key antibiotics used in the treatment of MRSA infections: vancomycin and linezolid. Although linezolid is one of the newest antibiotics, MRSA quickly became resistant to it according to that report (Arias et al, 2008). Due to the ability to rapidly develop tolerance to new antibiotics, and to produce many types of toxins, MRSA is currently known as one of the most serious pathogens in hospitals.

Coagulase-Negative Staphylococci

Staphylococci are conveniently divided into two groups based on the production of the enzyme coagulase. These are called coagulase-positive staphylococci (CPS) and coagulase-negative staphylococci (CNS). Coagulase is the enzyme that acts with a blood plasma factor to convert fibrinogen to a fibrin clot (Ryan and Ray, 2010). *Staphylococcus aureus*, including MRSA, is classified as a CPS. The CNS organisms are known as starter cultures for fermented food products and as opportunistic pathogens rather than as frank pathogens. For instance, *Staphylococcus succinus* and *Staphylococcus equorum* are both classified as CNS and are either associated with foods, or play a major role in the food processing industry. One of main advantages of starter cultures in food processing is that the fermentation and the ripening process can be carried out under controlled conditions (Zell et al, 2008). The CNS organisms are also known as normal flora of warm-blooded animals such as birds and mammals, including humans (Irlinger, 2008 and Place et al, 2002). For this reason, most CNS strains are thought of as being beneficial microorganisms rather than virulent pathogens.

However, some CNS strains can cause severe infections, especially in immunocompromised people, and are often difficult to treat because they are relatively antibiotic-resistant (Zell et al, 2008). For example, the oxacillin resistance of CNS lies between 70% and 80% according to reports from different parts of Europe (Diekema et al, 2001). Similar rates were also observed in the USA, Canada, and Latin America. In a report by Dar et al, it was shown that 22.5% of CNS isolates from 750 human subjects were resistant to methicillin (2006). Furthermore, an antibiotic-induced SOS response

promotes horizontal dissemination of pathogenicity island-encoded virulence factors in staphylococci (Ubeda et al, 2005). Those virulence factors include toxins such as hemolysins α,ß,γ, and δ, leukocidin, exfoliative toxins A and B, toxic shock syndrome toxin-1 (TSST-1), and a family of emetic pyrogenic superantigens (Zell et al, 2008).

In previous work in our lab, sixteen Gram-positive vancomycin-resistant cocci strains were isolated from the oral cavities of birds caught at the bird banding station on the campus of Fort Hays State University. These birds included eight American Robins (*Turdus migratorius*), three Orange-Warblers (*Vermivora celata*), and one each of Carolina Wren (*Thryothorus ludovicianus*), Least Flycatcher (*Empidonax minimus*), Swainson's thrush (*Catharus ustulatus*), and Mourning warbler (*Oporornis philadelphia*) (Bitner, 2008).

Those bacterial isolates were identified from the partial sequencing of the 16S rRNA gene. The closest match to fourteen of the isolates was *Staphylococcus succinus* AF004219, while the closest matches to the other two isolates were *Staphylococcus saprophyticus* and *Enterococcus gallinarum*. The MIC of vancomycin isolates was ≥ 256 µg /mL, and three of the isolates were shown to be coagulase-positive by the tellurite glycine agar method (Bitner, 2008).

In this study, I assayed those sixteen previously isolated Gram-positive vancomycin-resistant cocci for the presence of *van* genes and the ability to produce biofilm. I found *van* genes, *van*A, *van*B, and *van*C in several of these organisms. In addition, some of the isolates were also shown to have the ability to produce biofilm, which may be considered a significant virulence factor. However, the CNS strains are

usually not major pathogens, as mentioned earlier. A major concern of the CNS strains is that they could transfer virulence factors to other bacteria by horizontal gene transfer, leading to wide dispersal. Since the CNS are staphylococci, the virulence factors could be easily transferred to other more virulent *Staphylococcus* strains, such as MRSA.

Another major concern raised by this study is that the vancomycin-resistant organisms were isolated from birds that are commonly found all over North, Central, and parts of South America (Aldrich and James, 1991). The extensive habitat of these animals could allow them to serve as vectors to widely disseminate vancomycin-resistance genes in the environment. To the best of my knowledge, this is the first report of *van* genes being found in a staphylococcal species other than *Staphylococcus aureus* and the first report of *van*B in a bacterial genus other than *Enterococcus*.

MATERIALS AND METHODS

Organisms For This Study

I focused on the 16 vancomycin-resistant Gram-positive cocci previously isolated from wild song birds at the bird banding station at Fort Hays State University (Bitner, 2008).

Genomic DNA Isolation

Pure cultures of the bacteria were inoculated in 4.0 mL of tryptic soy broth (Difco, Detroit, Michigan, USA) and incubated for 12 hours at 37° C. Bacterial genomic DNA was isolated from the overnight cultures by using the protocol from Ausbel et al. (1997). The steps of the protocol were performed as follows: 1) 1.5 mL of overnight culture were centrifuged at 12,000 rpm for two minutes and the supernatant was removed; 2) The pelleted cells were resuspended in 567 µL of TE buffer (10mM Tris·Cl, and 1mM EDTA) buffer by repeated pipetting; 3) 30 µL of 10% SDS and 3µL of 20 mg/mL proteinase K were added into the mixture and the mixture was incubated at 37° C for one hour; 4) 100 µL of 5M NaCl were added and mixed thoroughly and 80µL of CTAB/NaCl solution (10% hexadecyltrimethyl ammonium bromide (CTAB)/0.7M NaCl) was added into the mixture and the mixture was incubated at 65 °C for 10 minutes; 5) 780 µL of chloroform/isoamyl alcohol (24:1) was added, mixed, then centrifuged at 12,000 rpm for five minutes; 6) the aqueous (top) layer was transferred to a fresh 1.5 mL centrifuge tube and 600 µL of phenol/chloroform/isoamyl alcohol (25:24:1) were added, mixed, and

centrifuged at 12,000 rpm for five minutes; 7) the aqueous layer was transferred into a fresh 1.5 mL microcentrifuge tube and 300 µL of isopropanol were added and mixed until DNA precipitated; 8) the mixture was centrifuged at 12,000 rpm for five minutes and the supernatant was removed; 9) the pelleted DNA was washed carefully by adding 1 mL of 70% ethanol then centrifuged at 12,000 rpm at five minutes; 10) the supernatant was removed and the pelleted DNA was dried by vacuum centrifugation until all traces of ethanol had been removed. The pellet was resuspended in 100 µL of sterile water and stored at -20° C. The final product was confirmed by agarose gel electrophoresis by using 10 µL of purified DNA and 3.0 µL of orange G loading dye. A 1.0% agarose gel in 1X TAE buffer (0.04 M Tris-Acetate and 0.001 M EDTA) was used with electrophoresis conditions of 90 volts for one hour. Samples were run next to the 1kb DNA ladder (Promega Corporation, Madison, Wisconsin). The agarose gel was stained with ethidium bromide, and viewed by using the Gel Logic 100 gel documenter (Eastman Kodak Company, Rochester, New York).

Polymerase Chain Reaction

The presence of *van* genes was detected by the polymerase chain reaction (PCR), using the extracted genomic DNA as the template, and specific primers for the *vanA, vanB, vanC*, and *vanD* genes. The primers used for PCR were EA1 (*vanA* forward), EA2 (*vanA* reverse), EB3 (*vanB* forward), EB4 (*vanB* reverse), EC5 (*vanC* forward), EC8 (*vanC* reverse), ED1 (*vanD* forward), ED2 (*vanD* reverse) (Depardieu et al, 2004b) (Table 1). The annealing temperatures for the reactions were based on the protocol

followed by Depardieu et al (2004b), which was 54° C. The following components for PCR were combined in a 0.5mL centrifuge tube: 39.5 µL sterile water, 5.0 µL standard *Taq* reaction buffer, 1.0 µL deoxynucleotide solution (dNTP) mix (containing deoxyadenosine triphosphate, deoxycytosine triphosphate, deoxyguanine triphosphate, and deoxythymine triphosphate, each at a 10 mM concentration), 0.5 µL bovine serum albumin, 0.5 µL Triton X-100, 0.5 µL forward primer, 0.5 µL reverse primer, 2.0 µL template DNA, and 0.5 µL *Taq* DNA polymerase. The PCR reaction was performed in a thermocycler using the following conditions: 94° C for three minutes, 40 cycles of 94° C for one minute, 54° C for 1 minute, and 72° C for 1 minute; the final elongation step was 72° C for seven minutes.

Detection of *van* Genes

The presence of PCR products was verified using agarose gel electrophoresis, using 20 µL of amplified DNA and 3.0 µL of orange G loading dye per well. A 3.0% agarose gel in 1X TAE buffer was used with electrophoresis conditions of 90 volts for 100 minutes. Samples were run next to the 100bp DNA ladder (Promega, Corporation, Madison, Wisconsin). The agarose gel was stained using the ethidium bromide, and viewed by using the Gel Logic 100 documentation system (Eastman Kodak Company, Rochester, New York). The amplified vancomycin resistance genes were compared to positive control PCR products from bacterial strains known to carry various *van* genes. I used *Enterococcus faecalis* SFV1 as the positive control for *van*A from Dr. Susan Flannagan at the University of Michigan (Ann Arbor, Michigan), *Enterococcus faecalis*

V583 as positive control for *van*B, and *Enterococcus gallinarum* ATCC 49579 as the positive control for *van*C from Dr. Ludek Zurek at Kansas State University (Manhattan, Kansas).

Crystal Violet Assay

One method used for the detection of biofilm was Crystal Violet Assay (CVA) (Toledo-Arana et al, 2001). Pure cultures of each organism were inoculated in 4.0 mL of TSB (Difco, Detroit, Michigan, USA) and grown for 12 hours at 37°C. *Staphylococcus epidermidis* ATCC 35984 and *Staphylococcus epidermidis* ATCC 12228 were used as positive and negative controls respectively in this assay. The steps of the protocol were performed as follows: 1) 200 µL of the bacterial cell suspension were used to inoculate a well in a sterile 96-well polystyrene microtiter plate (Fisher, Hanover Park, Illinois, USA). After 24 hours of incubation at 37°C, the bacterial suspension was removed; 2) The wells were gently washed three times with 200 µL phosphate-buffered saline (PBS); 3) The wells were dried at 50-60° C for one hour using Isotemp Vaccum Oven Model 280A; 4) The wells were stained with 200µL of 1% aqueous crystal violet for 20 minutes; 5) The wells were rinsed with deionized water; 6) The wells were fixed by ethanol-acetone (80:20, vol/vol); 7) The optical density at 550-590nm was determined using a microtiter plate reader (Sunrise, Salzburg, Austria).

Scanning Electron Microscopy

Specimens were prepared for scanning electron microscopy as described in Thurlow and Gillock (2005). Briefly, specimens were grown overnight in trypticase soy broth at 37°C. Samples were vacuum filtered through 0.45 μm HA filters **(**Millipore Corporation, Billerica, Massachusetts**)**. Filters containing the specimens were fixed in 0.15 M cacodylate buffer and 1% glutaraldehyde overnight. Filters were washed in a graded series of 95% ethanol and sterile distilled water for 15 minutes at each step (10% ethanol, 25% ethanol, 50% ethanol, 75% ethanol, 100% ethanol). Samples remained in ethanol for at least 24 hours. The filters were then dried in two steps by using hexamethyldisalazane (HMDS) and 95% ethanol (Step 1, 50% and HMDS 50% ethanol for 15 minutes: Step2, 100% HMDS until completely evaporated). Filters containing specimens were cut and mounted on a brass plate by using silver cement. The brass plates were fixed to an aluminum stub using silver cement. The specimens were coated with a layer of gold-palladium in a Pelco sputter coater for 1 minute. Specimens were viewed using ISI SX-30 scanning electron microscope (Topcon America Corporation, Paramus, New Jersey).

RESULTS

van Gene Detection by PCR

The PCR assay indicated that five of the 14 *Staphylococcus succinus* isolates carried the *van*A gene alone (Figure 6 and Table 2). In these organisms, PCR products of 732 bp in length were detected, clearly indicating the presence of the *van*A gene. In Figure 6, positive results from *Staphylococcus succinus* isolates V22 and V45 are shown. One of the *Staphylococcus succinus* isolates, V46, was demonstrated to carry three *van* genes; *van*A, *van*B, and *van*C (Figure 7 and Table 2). One isolate, *Enterococcus gallinarum*, or V49 appears to carry *van*C alone (Figure 8 and Table 2).

Biofilm Detection

Three of 14 *Staphylococcus succinus* isolates from American Robin, Mourning Warbler, and Least Flycatcher were shown to have the potential to form biofilm according to the results of the crystal violet assay. The density of crystal violet, as determined by absorbance at 620 nm, in the well for V56 is very similar to that of the well for the positive control, indicating V56 has the ability to form biofilm. (Figure 9 and Table 2). Biofilm was directly observed *via* scanning electron microscopy examination of one of the 14 *Staphylococcus succinus* isolates (Figure 10). The negative control strain for biofilm formation, *Staphylococcus epidermidis* ATCC 12228, clearly did not demonstrate the presence of biofilm (Figure 11). Conversely, biofilm formation was readily seen in the positive control strain for biofilm formation, *Staphylococcus epidermidis* ATCC 35984 (Figure 12).

DISCUSSION

Previous work indicated that 14 of 16 vancomycin-resistant bacterial isolates from six different birds species were *Staphylococcus succinus* according to 16S rRNA identification. One of the isolates was *Enterococcus gallinarum***,** while the other was *Staphylococcus saprophyticus* from the American Robin (Bitner, 2008).

Enterococci are a common nosocomial urinary tract and wound infection agent, and the third most common cause of nosocomial bacteremia in the United States. Many enterococci species are known as serious nosocomial pathogens since they can cause endocarditis and meningitis, and show intrinsic resistance against common antibiotics such as penicillin and fluoroquinolones. Due to the intrinsic resistance of enterococci to common antimicrobials, vancomycin was often used for the treatment, which ultimately led to vancomycin-resistant *Enterococcus* (VRE) (Cetinkaya et al, 2000). *Enterococcus gallinarum* is not commonly known as a serious nosocomial pathogen. However, this organism eventually became a vancomycin-resistant strain called vancomycin resistant *Enterococcus gallinarum* (VREG) which caused a nosocomial outbreak in a Colombian teaching hospital in 2004 (Contreras et al, 2008). In this case, the main clinical syndromes were bloodstream infections and surgical site infections. Unfortunately, Contreras et al could not detect the presence of any virulence-associated genes from VREG isolated from the outbreak (2008). However, they hypothesized that the VREG strain had the ability to produce serious infections and disseminate across the hospital environment since the outbreak did occur. They also did not detect the presence of any of the virulence factors from *Enterococcus faecalis* and *Enterococcus faecium* so they

suggest that VREG strains or unexplored pathogens carry virulence genes and facilitate horizontal transfer of antimicrobial resistance genes across bacterial populations (Contreras, 2008). Another study suggested that *Enterococcus gallinarum* might be the original strain which carries the *van*C gene intrinsically because of the degree of identity of the sequence (Gholizadeh and Courvalin, 2000) so that *Enterococcus gallinarum* from this study is not rare. However, the MIC against vancomycin of this isolate from this study was shown to be ≥ 256 μg/mL, which means that this isolate should have other mechanisms to tolerate such a high amount of vancomycin, since the usual resistance capability of *van*C-carrying organisms is 2-32 μg/mL (Cetinkaya et al, 2000).

One vancomycin-resistant isolate from this study was *Staphylococcus saprophyticus*, which is classified as a CNS, and is usually considered normal microflora, but can also cause medical risk. *Staphylococcus saprophyticus* may contribute to sausage aroma formation and help to prevent off-flavor during normal sausage ripening, but this strain also causes acute urinary tract infection (UTI) (Irlinger, 2008) and can be an agent of nosocomial and bloodstream infections (Zell et al, 2008). This strain accounts for up to 42% of all UTI in young women (Eiff et al, 2002). Several studies have reported that some *Staphylococcus saprophyticus* strains are vancomycin-resistant (Alamo et al, 1999). To my knowledge, however, this is the first report of a *Staphylococcus saprophyticus* strain which carries the *vanA* gene and has a vancomycin MIC of ≥ 256 μg/mL.

Most of the vancomycin-resistant bacterial isolates previously found in wild songbirds by Bitner were *Staphylococcus succinus* (2008). This microorganism is

normally associated with foods or plays a major role in the food processing industry, and is not usually considered a pathogen (Resch et al, 2008).

However, the opportunistic CNS can cause severe infections since they are relatively muti-drug resistant, as mentioned previously. In a study by Resch et al, they chose 21 antibiotics for assessment of resistance in the CNS organimsms *Staphylococcus succinus* and *Staphylococcus xylosus* (2008). They showed 90% of the assayed strains of these organisms exhibited resistance to the chosen antibiotics. Furthermore, some of the *Staphylococcus succinus* strains were resistant to up to four different antibiotics. However, none of their *Staphylococcus succinus* strains were resistant to vancomycin. They also observed differences in antibiotic resistance in CNS organisms isolated from different foods. Of the CNS organisms isolated from foods, 87% of those from hard and soft cheeses, 83% from sausage, and 93% from meat starter cultures exhibited high levels of resistance, whereas only 19% of the CNS organisms isolated from fermented fish were resistant (Resch et al, 2008). These investigators could not determine what factors affect the tolerance of antibiotic resistance in different foods. The MIC against vancomycin of most of the *Staphylococcus succinus* strains from this study was ≥ 256 μg/mL, indicating they are clearly vancomycin resistant, since the NCCLS (National Committee for Clinical Laboratory Standards) considers vancomycin-resistant microorganisms are those in which the MIC is above 32 μg/mL (Walsh and Howe, 2002).

Furthermore, some of the isolates in my study were found to be carrying either *van*A, *van*B, or *van*C. The *van*A and *van*B resistance genes are both inducible and have only been reported to exist in plasmid DNA (Courvalin, 2006). *van*A overcomes

vancomycin up to 1000 μg/mL and teicoplanin, in the same antibiotic group of vancomycin, up to 512 μg/mL, whereas *van*B overcomes vancomycin up to 1000 μg/mL and teicoplanin up to 1 μg/mL (Gholizadeh and Courvalin, 2000). As mentioned in the introduction section, vancomycin is one of the critical antibiotics for today's antibiotic treatment, but the high MIC of these resistant organisms are absolutely toxic for human since vancomycin is a strong antibiotic with many potential side effects. The ingestion of 18.0-47.0 μg/mL of vancomycin during treatment was observed to cause tinnitus, rash, neutropenia, and possible nephrotoxicity as side effects (Levine, 2006).

Three isolates from my study were shown to cause biofilm formation by CVA, which is known as a serious virulence factor because biofilm treatment is limited and is the leading cause of infection related to implanted medical devices (IMDs) (McCann et al, 2008). The biofilm adheres to abiotic objects such as IMDs and functions to cover the bacteria so that they can grow on the surface of the objects, while avoiding the host immune system and antibiotics. In some instances, biofilm-encased bacteria are dislodged into the circulatory system, which causes bacteremia. One of the major biofilm formation species is *Staphylococcus epidermidis*. In the case of *Staphylococcus epidermidis*, antibiotics might kill planktonic bacteria shed from the biofilm surface, but they fail to eradicate those embedded within the biofilm, which can then subsequently act as a reservoir for recurrent infection. Following antibiotic treatment, a minority of drug-resistant bacteria survive that repopulate the biofilm, with the survivors becoming much more antibiotic resistant (McCann et al, 2008). To overcome colonization and infections, many researchers and manufacturers have explored various surface technologies using

antimicrobial biomaterials. However, several researchers have raised concern that antimicrobial loading into a device by coating or immersion is a major driving force for the generation of resistance. To date, the only way to avoid the problems of creating resistant strains is the surgical removal of biofilm-coated objects. Bacteremia caused by biofilm-forming bacterial strains is a significant medical risk in hospitals (McCann et al, 2008). To the best of my knowledge, this is the first report of any *van* genes being found in a staphylococcal species other than *Staphylococcus aureus* as well as the first report of the *van*B in a bacterial genus other than *Enterococcus*.

Another key concern raised from this study is the isolation of extremely antibiotic-resistant organisms from highly mobile wild songbirds. The majority of birds which carried the highly vancomycin-resistant bacteria from this study were American Robins, which are migratory and cover most of North, and portion of Central, and parts of Northern South America (Aldrich and James, 1991). Since many American Robins appear to carry *Staphylococcus succinus*, it is conceivable that the bacterium is a normal flora microorganism of the American Robin. If this is the case, the American Robin could act as a vector animal to spread vancomycin-resistance genes over a wide range. Birds acting as vector animals for pathogens are not unusual. There are numerous examples of organisms such as bacteria, viruses, fungi, and parasites being spread over wide distances by birds (Tsiodras et al, 2008). For instance, avian influenza and West Nile Virus (WNV) are well known to be transported by birds. The 2009 outbreak of H1N1 influenza A is a particularly widely publicized example. In that case, the birds were also acting as media

animals, which facilitated the alteration of the viral antigens, causing antigenic shift, and creating more virulent viruses (Gatherer, 2009 and Patel et al, 2010).

Since bacteria freely participate in horizontal gene transfer, it is possible that the relatively innocuous highly vancomycin-resistant isolates from this study could transfer resistance genes to other more virulent microorganisms such as MRSA. According to Resch et al, CNS strains could have a high incidence of transfer of antibiotic-resistance genes to other staphylococci because of close phylogenetic relationships (2008). MRSA is a significant pathogenic agent in hospitals. It has had a severe impact upon the nosocomial infection mortality rate. The average mortality rate of MRSA from meta-analysis was ~36% compared to a mortality rate of ~24% from septicemia caused by methicillin-susceptible *Staphylococcus aureus* (MSSA). Furthermore, seven of the studies quoted MRSA bacteremia mortality rates over 50%, and two of these were over 80%. In the case of the patients who are infected with vancomycin-intermediate *Staphylococcus aureus* (VISA), the mortality is even higher (78%) (Dancer, 2008).

Additional research is needed to verify whether free-living wild song birds are really vector animals for vancomycin-resistance genes and whether vancomycin-resistant *Staphylococcus succinus* should be considered normal flora in the American Robin. Further research is also required to identify and understand the mechanisms of vancomycin-resistance used by bacteria in the absence of *van* genes or biofilm formation, as was seen in isolates V47, V67, V166, V170, V183, and V186 (Table 2). It is possible that these organisms are utilizing a cell wall thickening mechanism, or could be using a vancomycin-resistance pathway not yet reported in the literature.

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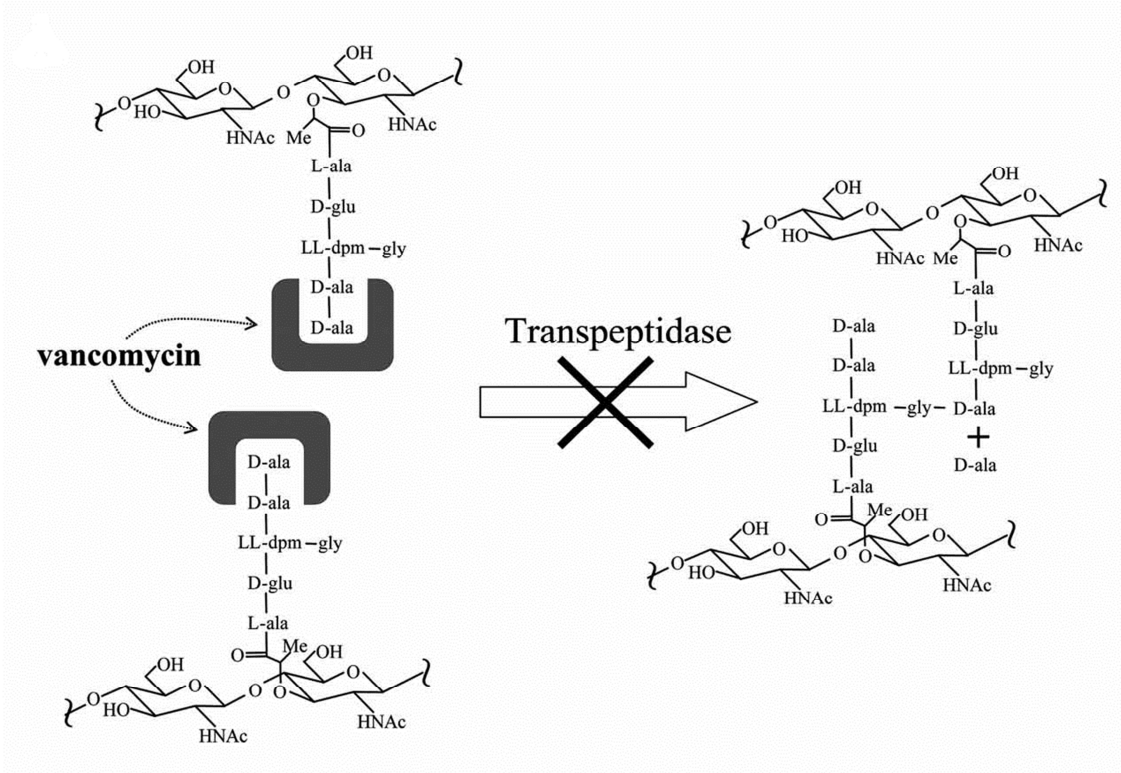


Figure 1. Transpeptidase and the mode of action of vancomycin. Transpeptidase recognizes the sequence D-Alanyl-D-alanine (D-Ala-D-Ala) at the end of the pentapeptide chains, cleaves off the terminal alanine and joins the remainder to the branch of a stem peptide from an adjacent polysaccharide chain. Vancomycin binds the D-Ala-D-Ala terminus. This interaction blocks formation of mature peptidoglycan, denying trandpeptidase access to its substrate. (From Hong et al, 2008).

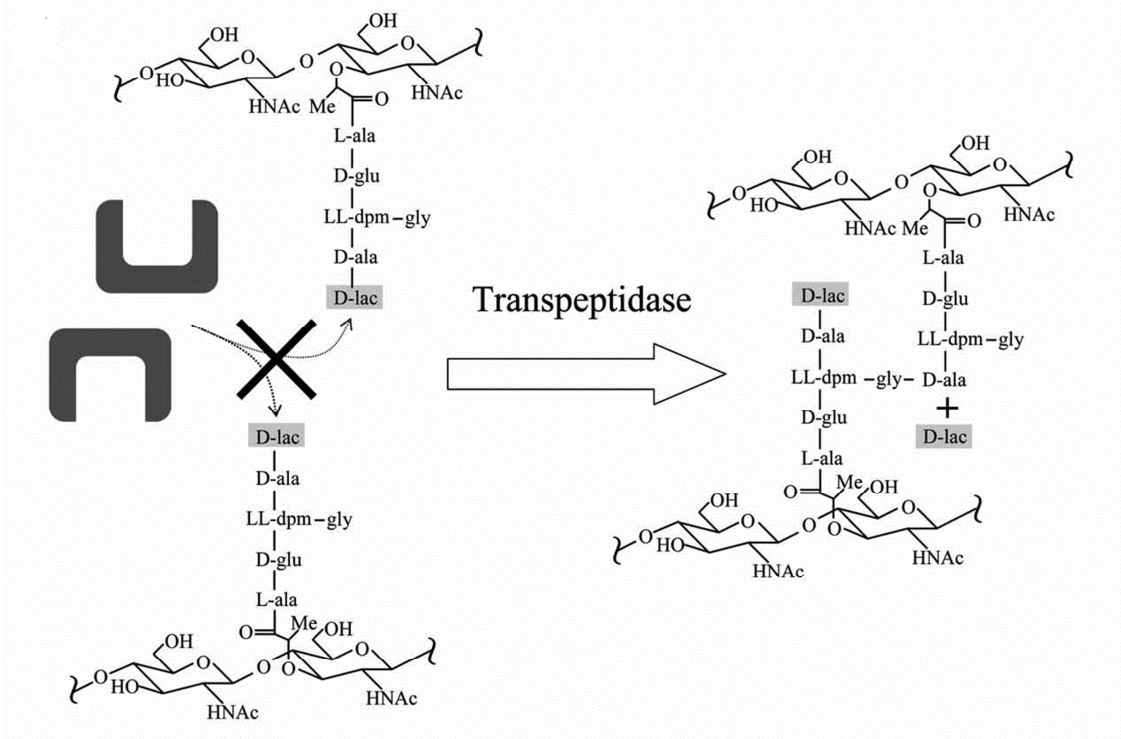


Figure 2. The mechanism of vancomycin-resistance in bacteria which carry *van* genes. Vancomycin resistant bacteria change the sequence of dipeptide to D-Alanyl-D-Lactate (D-Ala-D-Lac) at the end of the pentapeptide chain. This change lowers the affinity between vancomycin and the dipeptide. (From Hong et al, 2008).

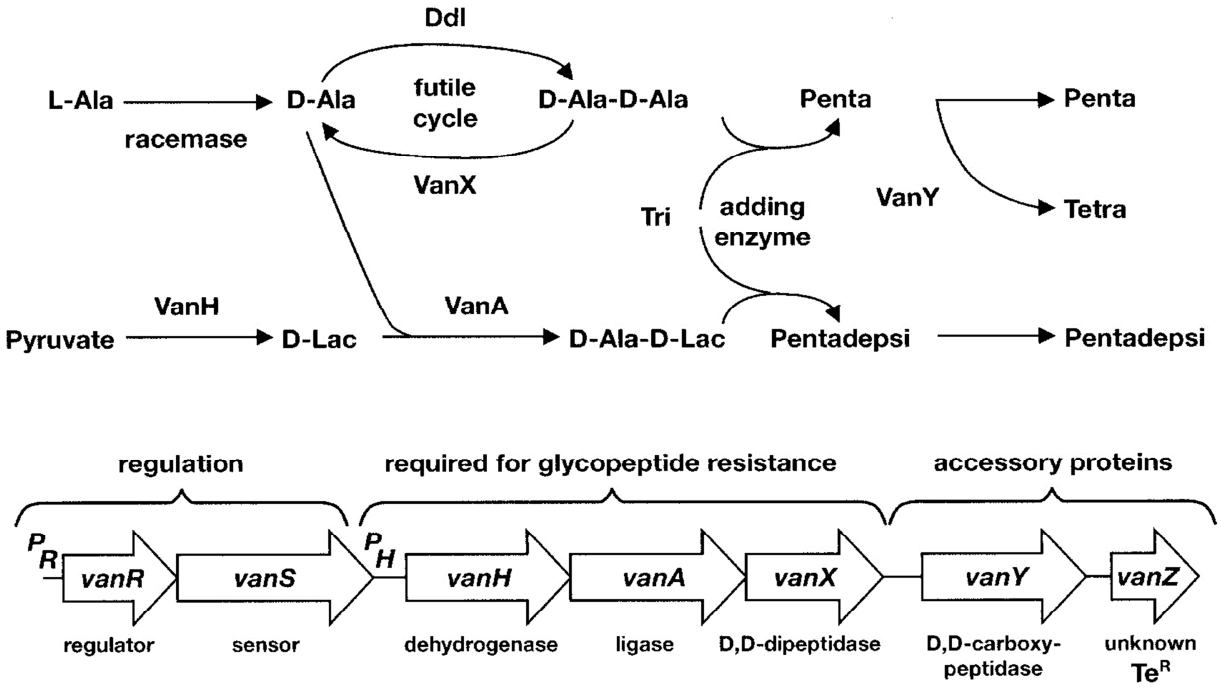


Figure 3. *van*A-type glycopeptide resistance. Top: Mechanism of synthesis of peptidoglycan precursors in a *van*A-type resistant strain. Bottom: *van*A-type glycopeptide resistance operon (From Courvalin, 2006).

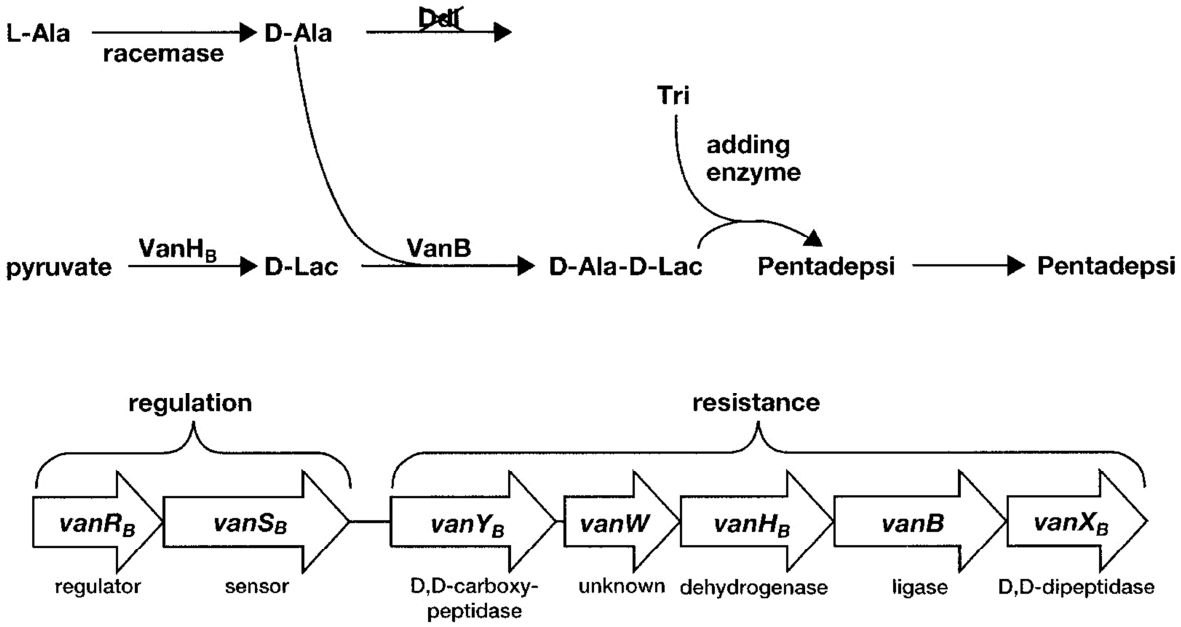


Figure 4. *vanB*-type glycopeptide resistance. Top: Mechanism of synthesis of peptidoglycan precursors in a *van*B-type resistant strain. Bottom: *van*B-type glycopeptide resistance operon (From Courvalin, 2006).

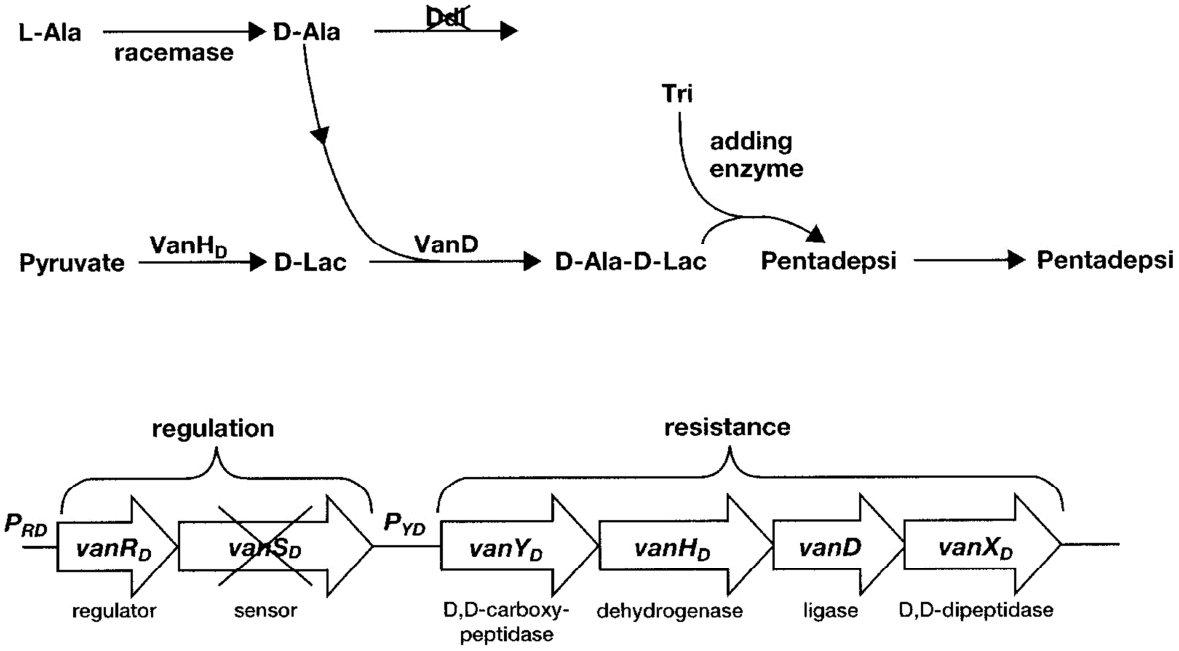


Figure 5. *van*D-type strain and vancomycin-dependent strain. Top: Synthesis of peptidoglycan precursors in a *van*D-type resistant strain. Bottom: Organization of the *van*D operon. Due to the inactivation of the host chromosomal D-Ala-D-Ala ligase (Ddl), the presence of vancomycin in the culture medium is required to induce expression of the resistance pathway, thus allowing cell wall synthesis (From Courvalin, 2006).

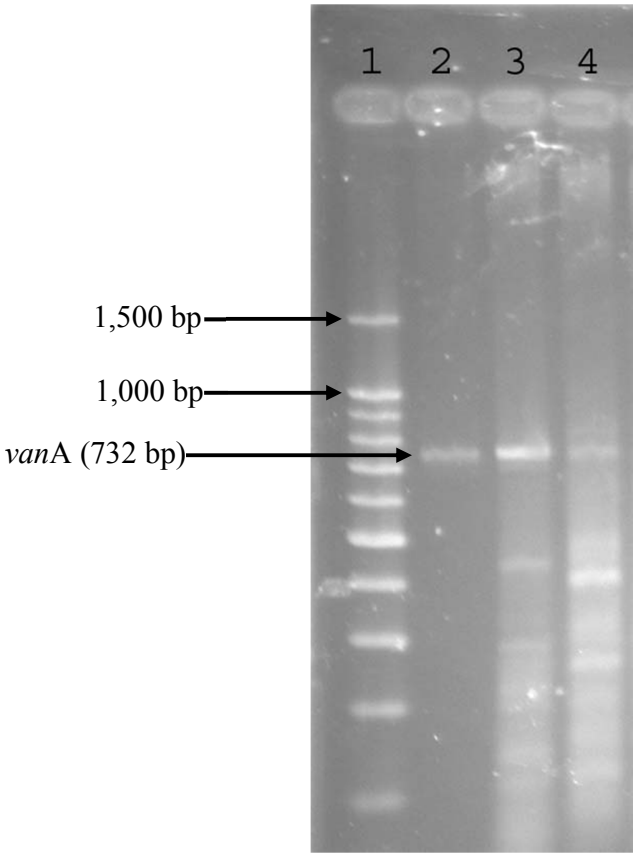


Figure 6. Agarose gel showing PCR detection of the *van*A gene. Lane1) 100 bp DNA ladder, Lane 2) *Enterococcus faecalis* SFV1 as positive control of *van*A, Lane3) *Staphylococcus saprophyticus* isolate V22, Lane 4) *Staphylococcus succinus* isolateV45. The bands in 100 bp ladder are (starting from the top):1,500, 1,000, 900, 800, 700, 600, 500, 400, 300, 200, and 100 bp.

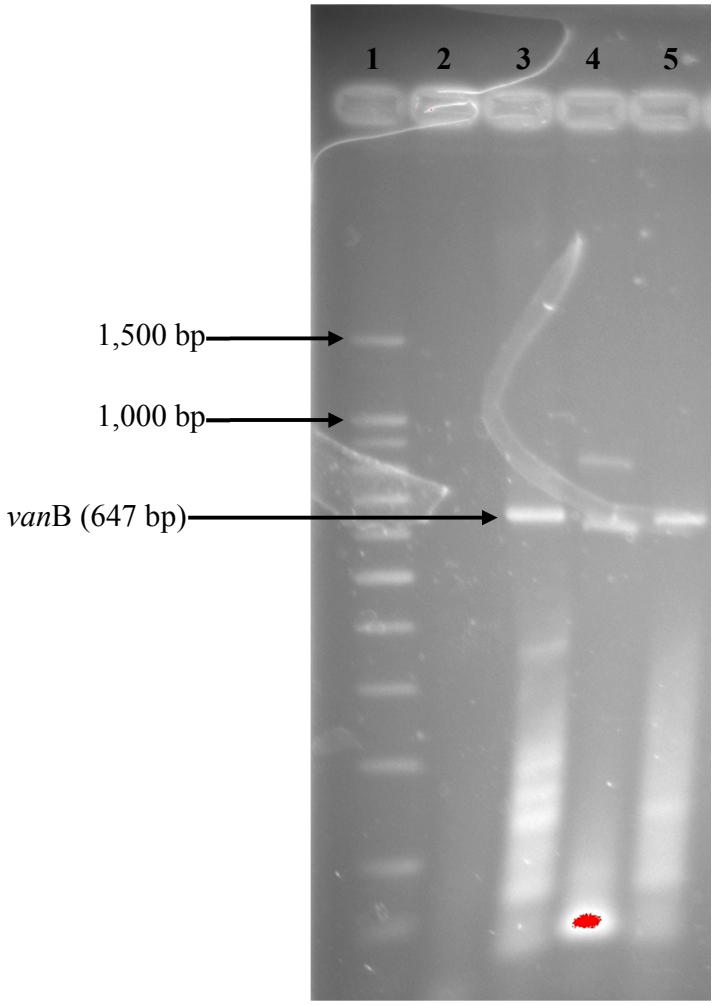


Figure 7. Agarose gel showing PCR detection of the *van*B gene. Lane 1) 100bp DNA ladder, Lane 3) *Enterococcus faecalis* V583 (Positive control), Lane 5) *Staphylococcus succinus* isolate V46.

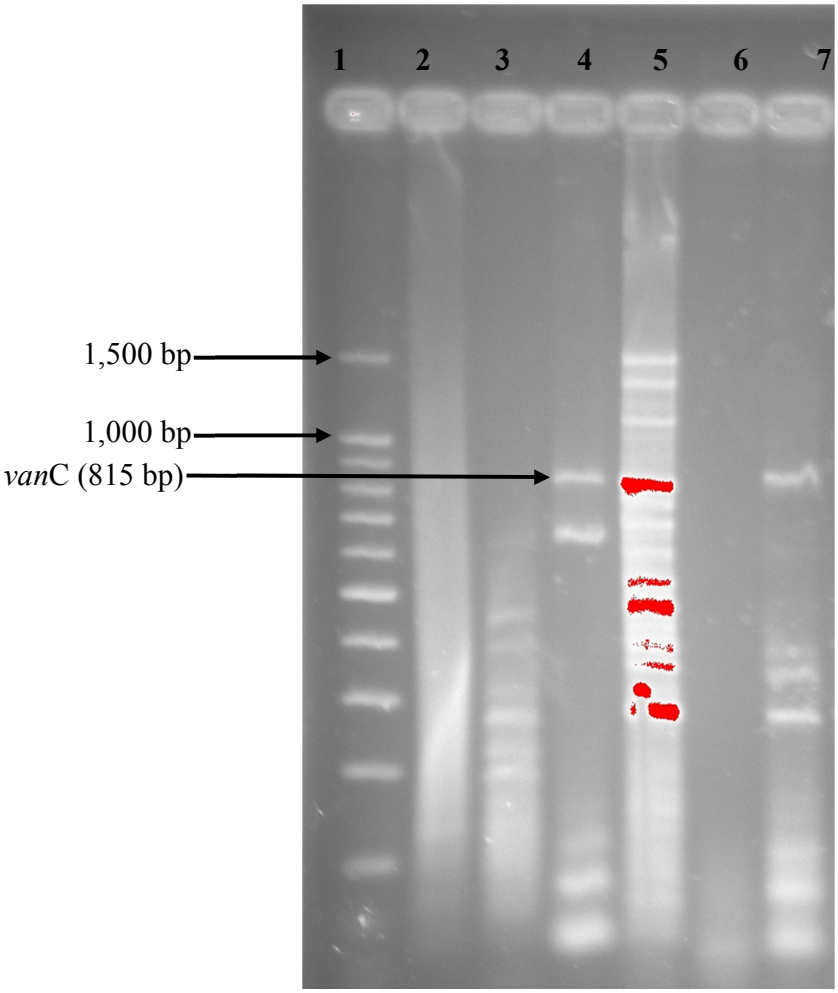
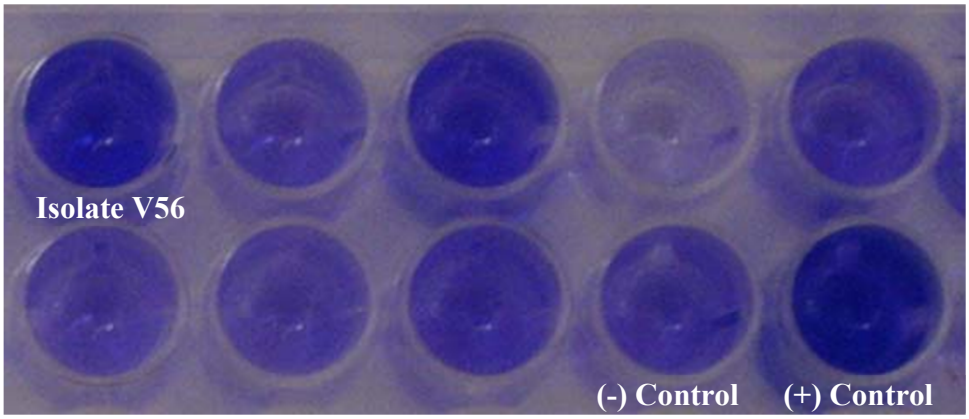


Figure 8. Agarose gel showing PCR detection of the *van*C gene. Lane 1) 100 bp DNA ladder, Lane 4) *Enterococcus gallinarum* ATCC 49579 (Positive control), Lane 7) *Enterococcus gallinarum* isolateV49.



	Isolates	V56	V154	V163	ATCC 12228	ATCC 35984
Absorbance at 620nm	Trial1	0.840	0.688	0.717	0.177	0.632
	Trial 2	0.801	0.631	0.647	0.162	0.647

Figure 9. Crystal Violet Assay for determination of biofilm formation. In this assay, *Staphylococcus epidermidis* ATCC 122288 was used as the negative control and *Staphylococcus epidermidis* ATCC 35984 was used the positive control. *Staphylococcus succinus* isolate V56 (indicated in the upper left well) clearly demonstrates biofilm formation. The table shows the absorbance of each well at 620nm. From this result, the isolate V56, isolate V154, and isolate V163 clearly form biofilm since the absorbance values of those wells are similar to the absorbance of ATCC 35984.

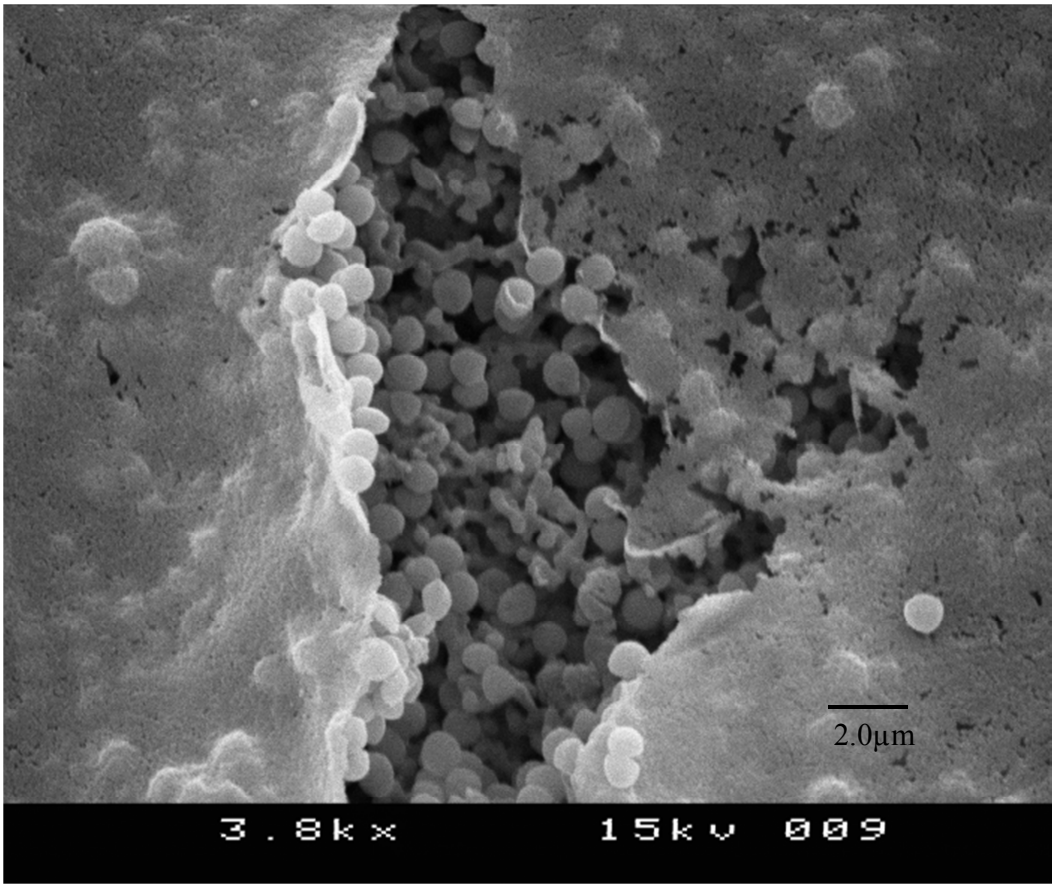


Figure 10. Scanning electron micrograph of *Staphylococcus succinus* isolate V56. The micrograph was taken at 3,800 X magnification. From this micrograph, V56 clearly forms a biofilm which covers the cocci-shaped bacterial cells. Scale bar equals approximately 2.0µm.

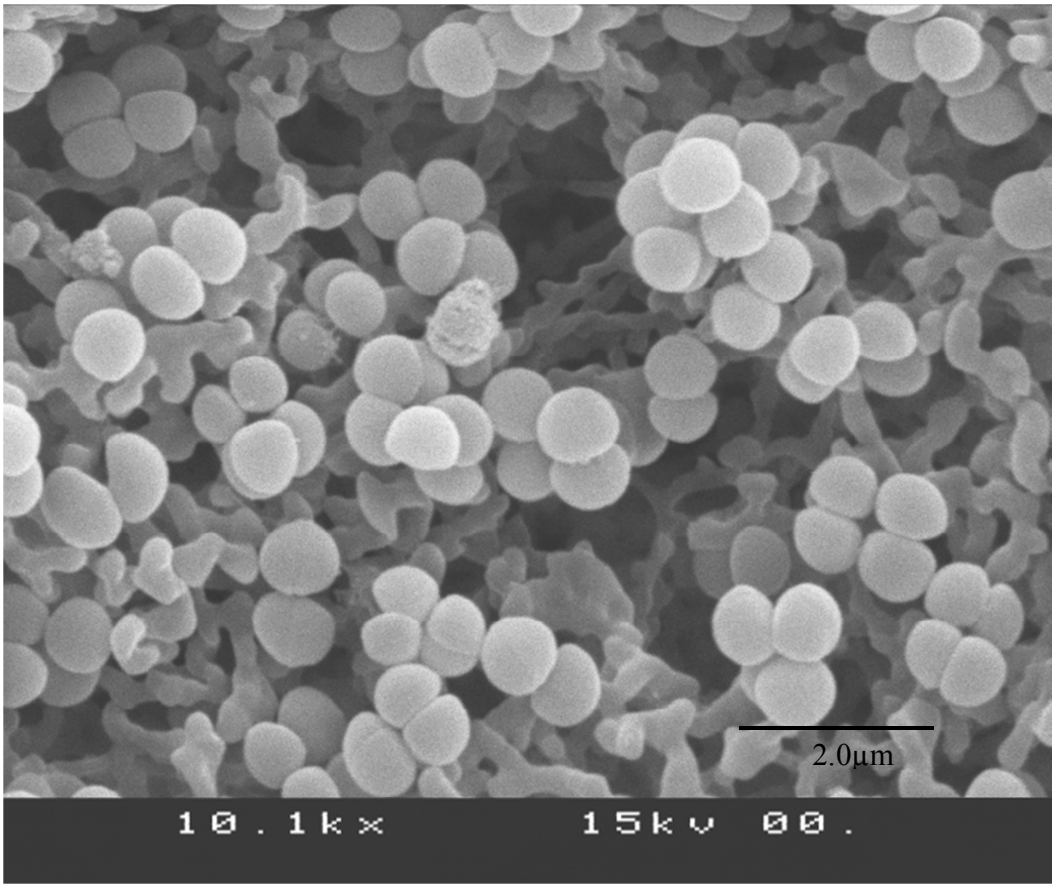


Figure 11. Scanning electron micrograph of the biofilm negative control strain, *Staphylococcus epidermidis* ATCC 12228. The micrograph was taken at 10,100 X magnification. From this micrograph, no biofilm structure is observed. Scale bar equals approximately 2.0µm.

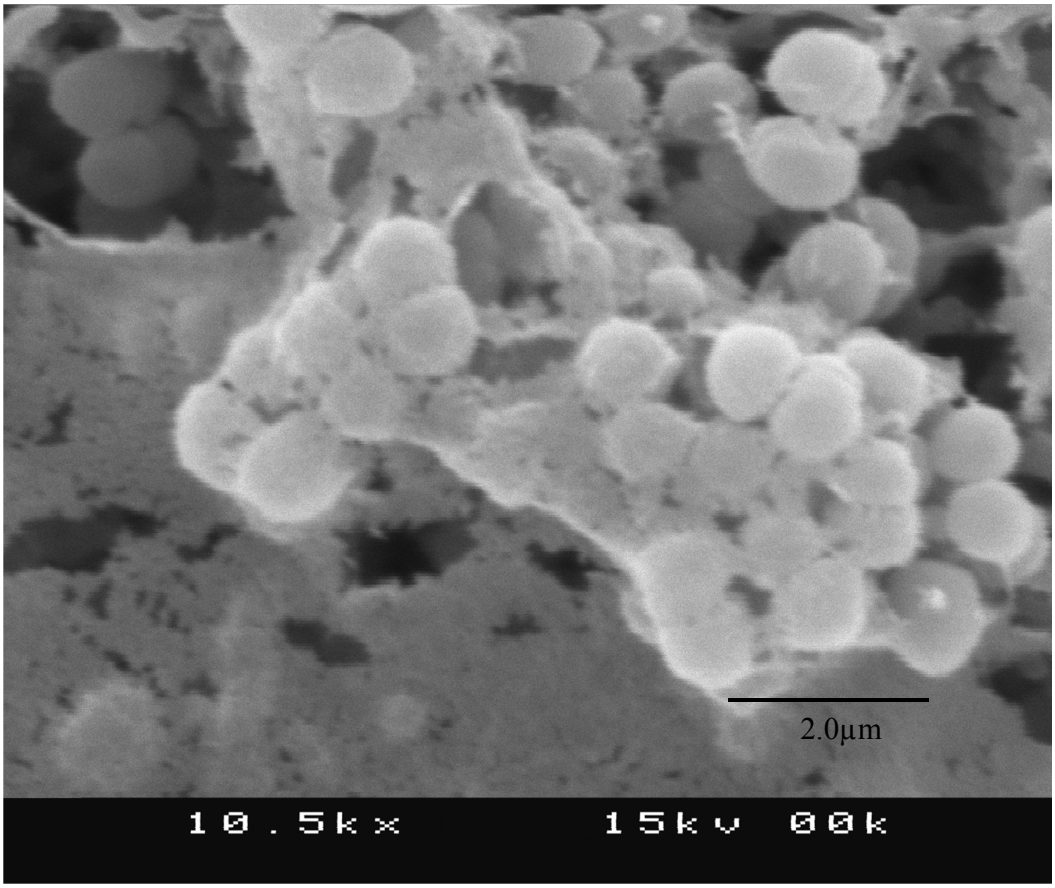


Figure 12. Scanning electron micrograph of the biofilm positive control strain, *Staphylococcus epidermidis* ATCC 35984. The micrograph was taken at 10,500 X magnification. From this micrograph, ATCC 35984 clearly forms a biofilm which covers the cocci-shaped bacterial cells. Scale bar equals approximately 2.0µm.

Gene	Primer	Sequence (5'-3')
van*A*	EA1(Forward)	GGGAAAACGACAATTGC
	EA2 (Reverse)	GTACAATGCGGCCGTTA
van*B*	EB3 (Forward)	ACGGAATGGGAAGCCGA
	EB4(Reverse)	TGCACCCGATTTCGTTC
van*C*	EC5(Forward)	ATGGATTGGTACTGGTAT
	EC8(Reverse)	TAGCGGGAGTGACCAGTAA
van*D*	ED1(Forward)	TGTGGGATGCGATATTCAA
	ED2(Reverse)	TGCAGCCAAGTATCCGGTAA

Table 1. Table showing the primers used for the PCR detection of the *van* genes in this study (From Depardieu 2004b).

Bacteria Species	Isolate Number	Coagulase	MIC (μg/mL)	Bird species	*van* genes	Biofilm
Staphylococcus saprophyticus	V22	-	≥ 256	American Robin	A	-
Staphylococcus succinus	V45	+	≥ 256	American Robin	A	-
Staphylococcus succinus	V46	-	≥ 256	American Robin	A, B, and C	-
Staphylococcus succinus	V47	-	≥ 256	American Robin	-	-
Enterococcus gallinarum	V49	-	≥ 256	American Robin	C	-
Staphylococcus succinus	V54	-	≥ 256	American Robin	A	-
Staphylococcus succinus	V56	-	≥ 256	American Robin	-	+
Staphylococcus succinus	V67	-	≥ 256	American Robin	-	-
Staphylococcus succinus	V185	-	1.5	Carolina Wren	A	-
Staphylococcus succinus	V163	-	≥ 256	Least Flycatcher	-	+
Staphylococcus succinus	V154	+	1.7	Mourning Warbler	-	+
Staphylococcus succinus	V187	-	≥ 256	Orange-crowned Warbler	A	-
Staphylococcus succinus	V166	-	≥ 256	Orange-crowned Warbler	-	-
Staphylococcus succinus	V170	-	≥ 256	Orange-crowned Warbler	-	-
Staphylococcus succinus	V186	+	2.0	Swainson's Thrush	-	-
Staphylococcus succinus	V183	-	≥ 256	Swainson's Thrush	-	-

Table 2. Summary of results showing species of vancomycin-resistant bacteria, isolate number, presence of coagulase activity, MIC of vancomycin activity, bird species the bacteria were isolated from, type of *van* genes detected, and presence of biofilm.